Differentiation of rat multipotent adult progenitor cells to functional hepatocyte-like cells by mimicking embryonic liver development

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Differentiation of stem cells to hepatocytes has industrial applications, as well as the potential to develop new therapeutic strategies for liver disease. The protocol described here, sequentially using cytokines that are known to have a role in liver embryonic development, efficiently differentiates rat multipotent adult progenitor cells (rMAPCs) to hepatocyte-like cells by directing them through defined embryonic intermediates, namely, primitive streak/mesendoderm/definitive endoderm, hepatoblast and hepatocyte-like phenotype. After 20 days, the final differentiated multipotent adult progenitor cell progeny is a mixture of cells, comprising cells with the characteristics of hepatoblasts and a smaller cell fraction with the morphological and phenotypical features of mature hepatocytes, as well as other mesodermal cells and some persistent undifferentiated rMAPCs. A detailed functional characterization of the stem cell progeny is also described; this should be used to confirm that differentiated cells display the functional characteristics of mature hepatocytes, including albumin secretion, glycogen storage and several detoxifying functions such as urea production, bilirubin conjugation, glutathione *S*-transferase activity and cytochrome activity.

INTRODUCTION

Primary hepatocytes are currently used for a number of applications: drug development, drug metabolism and drug toxicity studies (absorption, distribution, metabolism, excretion and toxicity studies, also known as ADMET studies), studies of hepatitis virus infection and creation of antiviral drugs, and investigations of new therapies for liver diseases, such as development of artificial liver devices and cell transplantation. However, hepatocytes isolated from human or animal liver can only be maintained in culture for a limited time, as they rapidly lose their phenotypic and functional characteristics. Stem or progenitor-derived functional hepatocytes have been suggested as a good alternative for primary hepatocytes, and the optimization of hepatic differentiation protocols is considered to be crucial for the further expansion of hepatocyte-like cells for applications in ADMET and therapeutic indications.

Multipotent adult progenitor cells (MAPCs)

Rodent multipotent adult progenitor cells (MAPCs) were first isolated in 2002 from the adherent fraction of the bone marrow (see **Box 1** for a brief summary of an updated isolation protocol[1]). Cultured rat (r)MAPCs have extensive proliferative (>100 population doublings) and multilineage differentiation capacity at the clonal level[2,3]. rMAPC clones express levels of *Oct4* between 10 and 60% of embryonic stem cells (ESCs). Phenotypically, rMAPCs are CD90 and MHC class II negative, have low levels of MHC class I and CD44 and are CD31 positive. The transcriptome analysis shows that rMAPCs express several ESC-associated transcripts (ECATs)[4], including *Oct4* and *Rex1*, as well as *nMyc*, *Lin28* and *Klf4*, known to have a role in the generation of iPSC, but do not express other ECATs, including *Nanog, Sox2* or the ESC gene *Esrrb*. Moreover, rMAPCs also express genes characteristic of primitive or visceral endoderm, such as XEN cells[5] or XEN-P cells[6], such as *Sox7*, *Sox17*, *Gata4*, *Gata6*, *Foxa2* and *Hnf1*β[3].

Since the description of MAPC in 2002 (ref. 7), different cell types with similar extended differentiation potency have been isolated from human or rodent tissues, including unrestricted somatic stem cells (USSCs)[8], human bone marrow–derived multipotent stem cells (hBMSCs)[9], marrow-isolated adult multilineage-inducible (MIAMI) cells[10], amniotic fluid-derived stem (AFS) cells[11], human fetal liver multipotent progenitor cells (hFLMPCs)[12], very small embryonic-like cells[13], pre-MSCs[14], human multipotent adult stem cells (MASCs)[15] and others. Similar to MAPCs, many of these cell populations display extensive expansion capacity and unexpected broad differentiation ability, as they can generate differentiated progeny beyond the boundaries of the tissue from which they were isolated. Some of the cell types described above also express *Oct4*, *Nanog* and *Sox2*, and, similar to ESCs, can generate cell types with the characteristics of cells from all three germ layers.

Hepatic differentiation of adult stem cells

MAPCs, pre-MSCs and MASCs generated from bone marrow[14–16], USSCs generated from UCB[17], AFS generated from amniotic fluid[18], and hFLMPCs generated from fetal liver[12] have been induced to differentiate into cells with hepatocyte-like cell characteristics *in vitro*. Although these cell types have a number of characteristics in common, the isolation and culture procedure, phenotype and potency are different. In 2002, we demonstrated that rMAPC lines could generate hepatocyte-like cells after a simple exposure to hepatocyte growth factor (HGF) and fibroblast growth factor (FGF)4, even though the different steps by which MAPCs acquired transcripts, proteins and functional attributes of hepatocytes were not described[16]. As the isolation techniques used for rMAPC isolation before 2002 differ from the ones we use now (see **Box 1**), the phenotype of rMAPCs used in the 2002 studies differed from that of the present rMAPC lines. Most notably, *Oct4* mRNA levels were >100-fold lower and 2002-undifferentiated rMAPCs did not express *Foxa2*, *Gata4* or *Gata6*. When we applied the cytokine mixture described previously[16,19] to induce differentiation of the more recently isolated *Oct4*^{high} rMAPC lines,

BOX 1 | ISOLATION OF RAT MULTIPOTENT ADULT PROGENITOR CELLS FROM BONE MARROW

Bone marrow aspiration from E18 or 4-week-old rats:
(i) Flush tibiae and femur with 15 ml of PBS containing 2% FBS under sterile conditions. Pass the flushed liquid through a 23-gauge needle.
(ii) Incubate the flushed bones for 30 min with PBS containing 0.2% collagenase and 0.02% DNase at 37 °C.
(iii) Filter both cell suspensions with a 40-μM cell strainer and centrifuge at 600*g* for 6 min at room temperature (20 °C).
(iv) Plate the cells in fibronectin-coated wells at 10^6 cells cm^{-2} in 2-ml MAPC expansion medium (**Table 3**).
High-density culture of cells for 1 month:
(v) For the first week of culturing, add 1 ml of medium every other day.
(vi) For the second week of culturing, change half of the medium every other day.
(vii) For the third and fourth weeks of culturing, trypsinize 1–2 times a week and replate at 80% confluence.
Column depletion of $CD45^+$cells with MACS microbeads:
(viii) After 4 weeks, trypsinize the cells, centrifuge and count. Label the cells with anti-rat CD45-PE antibody and anti-PE microbeads.
(ix) Run 3–5 × 10^6 cells through a 23–25-gauge needle through the MACS column at the rate of one drop every 4–5 s.
Subcloning, culture and screening for potential MAPC clones:
(x) Plate the eluted cells at five cells per well in a 96-well plate coated with fibronectin.
(xi) Change half of the medium every other day for 2 weeks.
(xii) After the second week, trypsinize colonies of 30–50 cells and expand them at low density without cell-to-cell contact.
(xiii) Maintain the cells at low density until small clusters of spindle/triangular small cells appear.
(xiv) Expand newly formed clusters at 300 cells cm^{-2}. Detection of cells with MAPC phenotype may require between 2–10 weeks of culture.
(xv) Screen the clones for *Oct4* by RT-qPCR or staining.

Adapted from Subramanian *et al.*[1].

hepatic genes were minimally induced. This led us to re-evaluate the culture conditions for liver differentiation.

The 20-day differentiation protocol described here is modeled on liver embryonic development (**Fig. 1**). To mimic Nodal/Cripto signaling and the canonical β-catenin activity involved in primitive streak (PS) and definitive endoderm (DE) formation, cells were first treated for 6 days with Activin-A and Wnt3a, respectively. Cytokines produced by the cardiac mesenchyme and the septum transversum mesenchyme to induce formation of hepatic endoderm from anterior endoderm were mimicked *in vitro* by the stimulation of cells with BMP4 and FGF2 for 4 d. A combination of FGFs (FGF1, FGF4 and FGF8) was used to mimic the growth factors that induce liver bud growth (4 d), and finally hepatocyte growth factor (HGF) and Follistatin were used to enhance the maturation of generated hepatoblasts into hepatocyte-like cells (6 days). The main difference between this protocol and the previously described protocol is the sequential exposure based on liver embryogenesis. Both cytokines used in the previous protocol (FGF4 and HGF) are incorporated into the current protocol, but are only used from day 10 onward, after two initial steps aimed at mimicking gastrulation and hepatic endoderm formation.

Evaluation of the success of the protocol

To monitor and evaluate the protocol efficiency, RT-qPCR and immunocytochemistry can be performed. A list of key genes to be evaluated is presented in **Box 2**, while the primer sequences we have successfully used are listed in **Table 1**. As a housekeeping gene, we have used *Gapdh*. We used the Qiagen RNeasy kit for RNA isolation as per the manufacturer's instructions, but alternative methods can be used. As positive controls for RT-qPCR, RNA from rat fetal liver at E15 and isolated adult rat hepatocytes are suitable.

For immunocytochemistry, a list of possible combinations of antibodies for staining at different stages is presented in **Box 2**, whereas the antibodies, isotypes and fixatives we use are listed in **Table 2**.

As functional analysis, we chose a combination of secretion (albumin, urea), storage (glycogen) and detoxification capacity (cytochrome P450, glutathione *S*-transferase (GST), bilirubin conjugation). Albumin secretion is detectable from day 14 onward. Spontaneous urea production can already be found from day 6, but urea production in response to ammonia (urea cycle) is detectable from day 10, becoming maximal by day 20. Many substrates are conjugated to GSH by GST, for example, 1-chloro-2,4-dinitrobenzene (CDNB). Therefore, the formation of GSH-CDNB is used as a quantitative assay to measure total GST activity. Subtypes of GST can be analyzed as well by using ethacrynic acid to measure fetal GSTπ activity and di-chloro-nitrobenzene for more mature GSTμ activity (protocol not shown).

Experimental design

The differentiation capacity of rMAPCs is unaffected by the passage number (no differences were seen for differentiations carried out between passages 17 and 35, equivalent to >100 population

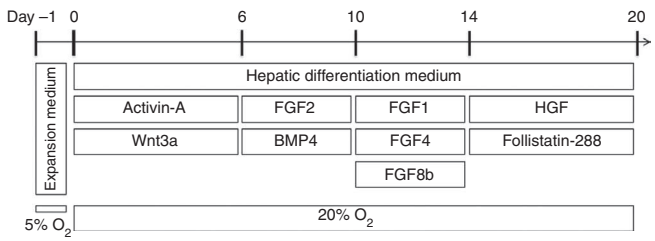


Figure 1 | Overview of the liver differentiation protocol.

BOX 2 | REAL-TIME RT-qPCR ANALYSIS OF TRANSCRIPTS

(Items in boldface type are detailed in **Fig. 3**.)
Undifferentiated MAPCs: *Oct4*, *Foxa2*, *Sox7*, *Sox17*
Primitive streak/mesendoderm: *Eomes*, ***Mixl1***, *Brachyury*, ***Gsc***, *E-cadherin*
Definitive endoderm: *Hex*, *Mixl1*, ***Cxcr4***, ***Tm4sf2***, *Lhx1*, *Gsc*, *Sox17*, *E-cadherin*
Hepatoblasts: ***Afp***, ***Ttr***, ***Krt19***, ***Alb***
Hepatocytes: ***Alb***, ***Aat***, ***Tat***, ***G6pc***, *Adh1C*, *ApoF*, *Mrp2*, *Cps-1*, *Arg-1*, *Pepck*, *Cx32*, *HepPar1*, *To*, *Cyp2b6*, ***Cyp1a2***, *Cyp7a1*, *Bsep*, *GlyS*, ***Factor V***
Liver-enriched transcription factors: *Hnf4α*, *Hnf1α*, *Hnf6*, *Prox1*, *Cebpα*, *Cebpβ*

Adapted from Sancho-Bru *et al.*[28].

doublings). rMAPCs with higher passage number do accumulate more genetic abnormalities; therefore, most of the cell cultures should be terminated around passage numbers 35–40.

Using the protocol described here, rMAPCs can be efficiently differentiated into PS/mesendoderm (ME)/DE, followed by the acquisition of a hepatoblast phenotype and finally the acquisition of mature hepatocyte characteristics. Freshly isolated rat hepatocytes should be used as positive control for functional assays. Alternatively, immortalized cell lines (such as HepG2) can be used, bearing in mind that gene expression and functionality may differ from primary hepatocytes. As negative control, undifferentiated rMAPCs (day 0) can be used.

Hepatocyte differentiation from rMAPCs as described here is still in an experimental phase and the impurity of the final progeny makes them not yet suitable for toxicological or preclinical studies. However, ongoing studies to scale the current protocols and to purify cells with mature hepatocyte features from the mixed progeny generated using the current protocol may ultimately yield rMAPC hepatocyte progeny with mature functional characteristics similar to those of hepatocytes (metabolization of ammonia to urea, inducible cytochrome activity, bilirubin conjugation), which may be used for *in vitro* screening purposes including ADMET studies.

Conclusion

A large number of protocols have been described to differentiate various types of mesenchymal stem cells into hepatocyte-like cells[20–22]. In most of these protocols, a sequential exposure or a combination of HGF, FGF and Oncostatin M is used. Obviously, the main focus of these protocols is the generation of hepatocytes, but only little emphasis to developmental milestones such as gastrulation has been paid. Because of the numerous differences between the protocols and the starting cell populations, it is nearly impossible to distinguish which protocol is the best or the most efficient. Besides the generation of functional hepatocyte-like cells, the main benefit of the protocol described here is that it also creates a model to study the early steps of liver embryogenesis. We have not yet determined whether the protocol described here also induces differentiation of MSCs or more primitive cells such as MIAMI, MASCs, BMSCs, USSCs and pre-MSCs among others into hepatocyte-like cells. However, we did demonstrate that, with minimal changes, the protocol that supports rMAPC differentiation into hepatocyte-like cells can also be applied for hepatic differentiation of pluripotent cells, such as human and mouse embryonic and induced pluripotent stem cells, suggesting that cells susceptible to hepatocyte differentiation using the protocol described here should have (near)pluripotent features.

TABLE 1 | Primers.

Genes	Forward sequence	Reverse sequence
Aat	5′-CAAACAAGGTCAGCCATTCTC-3′	5′-CAGCATCATTGTTGAAGACCC-3′
Afp	5′-ACCTGACAGGGAAGATGGTG-3′	5′-GCAGTGGTTGATACCGGAGT-3′
Alb	5′-TCTGCACACTCCCAGACAAG-3′	5′-AGTCACCCATCACCGTCTTC-3′
Cxcr4	5′-GGATGGTGGTGTTCCAGTTC-3′	5′-TCCCCACGTAATACGGTAGC-3′
Cyp1a2	5′-GTCCAGGAACACTATCAAGAC-3′	5′-ACTGTTTCAAATCCAGCTCC-3′
Factor V	5′-CAATGCCAGATGTAACAGTC-3′	5′-TGTCAATATAAGCCTGCATCC-3′
G6pc	5′-GATTCCGGTGCTTGAATGTC-3′	5′-AGGTGATGAGACAGTACCTC-3′
Gapdh	TaqMan Rodent GAPDh control reagents (Applied Biosystems, cat. no. 4308313)	
Gsc	5′-CCCGGTTCTGTACTGGTGTC-3′	5′-CCCACGTCTGGGTACTTTGT-3′
Krt19	5′-CCACACTACGCAGATCCAGA-3′	5′-ATGCTGAGCTGAGACTGCAA-3′
Mixl1	5′-GGGAAGATTTCCTCCATCGT-3′	5′-CTGAGAACCAGATGTACAGAC-3′
Tat	5′-GGAAGCTAAGGATGTCATTCTG-3′	5′-GACCTCAATTCCCATAGACTC-3′
Tm4sf2	5′-CTGAAACTGTATGCCATGTTCC-3′	5′-ATCTTTGCCGTTGTAGTTCTG-3′
Ttr	5′-CAGCAGTGGTGCTGTAGGAGTA-3′	5′-GGGTAGAACTGGACACCAAATC-3′

TABLE 2 | Antibodies.

	Company	Catalog no.	Dilution	Isotype
Primary antibodies				
Oct3/4 (N19)	Santa Cruz Biotech	SC8628	1:1,000	Goat IgG
Sox7	R&D Systems	AF2766	1:20	Goat IgG
Sox17	R&D Systems	MAB 1924	1:300	Mouse IgG_3
Foxa2	Santa Cruz Biotech	SC6554	1:200	Goat IgG
Alb	Dako	A0001	1:8,000	Rabbit IgG
Afp	R&D Systems	MAB 1368	1:4,000	Mouse IgG_1
Krt18 (N16)	Santa Cruz Biotech	SC31700	1:250	Goat IgG
Pepck (H-300)	Santa Cruz Biotech	SC32879	1:200	Rabbit IgG
Secondary antibodies				
Chicken anti-rabbit Alexa 488 (green)	Invitrogen	A21441	1:500	
Donkey anti-goat Alexa 488 (green)	Invitrogen	A11055	1:500	
Rabbit anti-mouse Alexa 488 (green)	Invitrogen	A11059	1:500	
Donkey anti-goat Alexa 555 (red)	Invitrogen	A21432	1:500	
Goat anti-mouse Alexa 555 (red)	Invitrogen	A21424	1:500	
Goat anti-rabbit Alexa 555 (red)	Invitrogen	A21429	1:500	
Hoechst (nuclear staining, blue)	Sigma	33258	1:2,000	
Isotypes (protein concentration as primary antibody)				
Mouse IgG_1	BD Biosciences	550878		
Mouse IgG_3	Sigma	I3784		
Goat IgG	Jackson Labs	JACK005-000-0020		
Rabbit serum	Dako	X0902		
Miscellaneous				
Fixation: 10% NBF	Sigma	HT501128		
Permeabilization: Triton X-100	Acros Organics	422355000		
Blocking: normal donkey serum	Jackson Labs	JACK017-000-121		
Mounting: ProMount Gold	Invitrogen	P36930		

NBF, neutral buffered formalin.

MATERIALS

REAGENTS

- Ammonium bicarbonate (NH_4HCO_3) (Sigma, cat. no. 09830), Step 22, option C only
- Anthrone (Sigma, cat. no. A-1631), Step 22, option B only
- Anti-PE MicroBeads (Miltenyi Biotec, cat. no. 130-048-801)
- Bilirubin (Sigma, cat. no. B4126)
- BSA (Sigma, cat. no. A9418)
- Cell strainer with 40-μm nylon mesh (BD Biosciences, cat. no. 352340)
- Chloroform for HPLC (LabScan, cat. no. PLC05___X) Step 22, option F only **! CAUTION** Irritating, danger of serious health damage by prolonged exposure through inhalation. Use in a well-ventilated area.
- Collagenase D (Roche, cat. no. 11-088-858-001)
- Cytokines (R&D Systems): Activin-A (338-AC), BMP4 (314-BP), EGF (2028-3G), FGF1 (232-FA), FGF2 (233-FB), FGF4 (235-F4), FGF8b (423-F8), Follistatin-288 (769-FS), HGF (294-HGN), PDGF-BB (220-BB) and Wnt3a (1324-WN)
- Dexamethasone (Sigma, cat. no. D2915)
- DMEM-low glucose (Gibco, cat. no. 31885)
- DNase (Sigma, cat. no. D-4263)
- ELISA Starter Accessory Kit (Bethyl, cat. no. E101-122), Step 22, option A only
- Fibronectin (Sigma, cat. no. F-0635)
- Fetal bovine serum (HyClone, cat. no. CH30160.03)
- Glutathione (GSH) (MP Biomedicals, cat. no. 02101814), Step 22, option E only
- Glycogen (Sigma, cat. no. G-0885), Step 22, option B only
- Growth factor-reduced Matrigel (BD Biosciences, cat. no. 354230)
- Insulin–transferrin–selenium liquid medium supplement (Sigma, cat. no. I-3146)
- Leukemia inhibitory factor (10^7 units ml^{-1}) (Chemicon, cat. no. ESG-1107)
- Linoleic acid–BSA (LA-BSA, 0.845 mg ml^{-1} LA, 100 mg ml^{-1} BSA) (Sigma, cat. no. L-9530)
- MACS separation CS column (Miltenyi Biotec, cat. no. 130-041-305)
- MCDB 201 medium with trace elements, L-glutamine and 30 mM HEPES (MCDB; Sigma, cat. no. M-6770)
- Methanol for HPLC (VWR BPH Prolabo, cat. no. 20837.320), Step 22, option F only

- Omeprazole (AstraZeneca, cat. no. M130064AB), Step 22, option D only
- PBS pH 7.4 w/o $CaCl_2$ and $MgCl_2$ (Gibco, cat. no. 10010-015)
- PE mouse anti-rat CD45 (BD Pharmingen, cat. no. 554878)
- Penicillin–streptomycin solution (Cellgro, cat. no. 30-002-CI)
- Phenobarbital (Sigma, cat. no. P1636), Step 22, option D only
- Phosphoric acid solution for HPLC (H_3PO_4) (Fluka, cat. no. 79626), Step 22, option F only
- P450-Glo CYP1A2 Assay (Promega, cat. no. V8771), Step 22, option D only
- QuantiChrom Urea Assay Kit (BioAssay Systems, cat. no. DIUR-500), Step 22, option C only
- Rat Albumin ELISA Quantitation Set (Bethyl, cat. no. E110-125), Step 22, option A only
- Rat MAPC (see **Box 1** for preparation details)
- Tetrabutylammonium hydrogen sulfate (TBA) for HPLC (Acros, cat. no. 42010), Step 22, option F only
- Tris-phosphate-EDTA buffer 0.8 M (Sigma, cat. no. T3154), Step 22, option F only
- Trypsin-EDTA 0.05% (Gibco, cat. no. 25300)
- Trypsin-EDTA 0.25% (Gibco, cat. no. 25200)
- White opaque 96-well plates (Thermo Scientific, cat. no. 15042), Step 22, option D only
- Xanthobilirubic acid (prepared according to the method of Grünewald *et al.*[23]), Step 22, option F only
- 1-Chloro-2,4-dinitrobenzene 99% (CDNB; Acros Organics, cat. no. 16051), Step 22, option E only **! CAUTION** Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment. Collect waste separately.
- 2-Mercaptoethanol 50 nM 1,000× (Gibco, cat. no. 31350)

EQUIPMENT

- Analytical nitrogen evaporator (Organomation, cat. no. N-EVAP 11250), Step 22, option F only
- BioPhotometer 8.5 mm (Eppendorf, cat. no. 6131 23861)
- Luminometer (Thermo-Labsystems Fluoroskan Ascent FL)
- Liquid chromatograph (Waters, cat. no. 995 photodiode + 2× Waters, cat. no. 515 HPLC pump)
- Microplate reader (Bio-Rad, model 3550)
- NucleoCounter + NucleoCassette (Chemometec)
- Spectrophotometer (Shimadzu, cat. no. UV-2101)
- Stericup with Millipore Express PLUS 0.22-μm filter (Millipore, cat. no. SCGP U05 RE)

REAGENT SETUP

Matrigel-coated plates Store Matrigel (10-ml bottle) at −20 °C. It is advisable to freeze down Matrigel in separate vials of smaller volume (for example, 400 μl) to avoid unnecessary freeze-thaw cycles. Matrigel polymerizes at room temperature (20 °C), hence it is necessary to thaw the bottle of Matrigel on ice and use frozen pipette tips and cryotubes. If thawed too quickly, Matrigel will partially polymerize and give rise to clumps.

Thaw one vial (400 μl) of Matrigel slowly on ice. Dissolve 400 μl of Matrigel in 20 ml of ice-cold PBS (final concentration 2%) and add 1 ml to every well of a 12-well plate or chamber slide. Incubate for at least 30 min at 37 °C or for 2–3 h at room temperature. Use immediately.

MCDB solution Dissolve one vial of MCDB 201 medium (17.7 g) in 1,000 ml of Milli-Q water in a flat-bottom Erlenmeyer flask. Stir with a magnetic stir bar until completely dissolved (yellow suspension). Measure the pH and adjust with NaOH or HCl dropwise until it equals 7.2. Filter the solution using Stericup filter. The solution can be stored at 4 °C for up to 6 weeks.

0.2% BSA-PBS solution Dissolve 100 mg of BSA in a final volume of 50 ml of PBS and filter using Stericup filter to produce a 0.2% BSA-PBS solution. Store at 4 °C for several weeks.

0.2% BSA-HCl solution Dilute 200 μl of HCl (stock concentration 2 M) in 100 ml of distilled water to produce a 4 mM HCl solution. Dissolve 200 mg of BSA in a final volume of 100 ml of 4 mM HCl solution. Filter using Stericup filter. Store at 4 °C for several weeks.

2% KOH solution Dissolve 2 g of KOH in a final volume of 100-ml methanol to produce a 2% solution. Store at room temperature for several weeks.

60 μM BSA solution Dissolve 198 μg of BSA in 50 ml of hepatic differentiation medium to produce a 60 μM BSA solution. Use immediately.

Activin-A solution Dissolve 5 μg (one vial) in 500 μl of 0.2% BSA-PBS to produce a 10-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

Anthrone solution Dissolve 2 mg of anthrone in 1,000-μl H_2SO_4 to produce a 0.2% solution (yellow color). Use immediately.

Bilirubin solution Dissolve 17.5 mg of bilirubin in 5 ml DMSO to produce a 6 mM stock solution. Dissolve 250 μl of stock solution in 50 ml of 60 μM BSA solution to produce a final 30 μM bilirubin solution. Use immediately.

BMP4 solution Dissolve 10 μg (one vial) in 1,000 μl of 0.2% BSA-HCl to produce a 10-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

CDNB solution Dissolve 30.4 mg of CDNB in 5 ml of ethanol to produce a 30 mM solution. Use immediately.

Dexamethasone solution Dissolve 100 mg (one bottle) in 1,000 ml of distilled H_2O to produce a 0.25 mM solution. Filter using Millipore Express plus a 0.22-μm filter. Store at −20 °C for several months.

EGF solution Dissolve 1 mg (one vial) in 5 ml of 0.2% BSA-PBS to produce a 200-ng μl^{-1} solution. Store at −80 °C for several months.

Expansion medium (Table 3) Store at 4 °C for 1 month.

FGF1 solution Dissolve 25 μg (one vial) in 2,500 μl of 0.2% BSA-PBS to produce a 10-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

FGF2 solution Dissolve 25 μg (one vial) in 2,500 μl of 0.2% BSA-PBS to produce a 10-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

FGF4 solution Dissolve 25 μg (one vial) in 1,000 μl of 0.2% BSA-PBS to produce a 25-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

FGF8b solution Dissolve 25 μg (one vial) in 1,000 μl of 0.2% BSA-PBS to produce a 25-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

Fibronectin solution Dissolve 2 mg in 4,000 μl of PBS to produce a 500-ng μl^{-1} stock solution. Dilute 100 μl of stock solution in 500 ml of PBS to produce a final 100-ng ml^{-1} solution. Filter using Millipore Express plus 0.22-μm filter. Store at 4 °C for several weeks.

Follistatin solution Dissolve 25 μg (one vial) in 2,500 μl of 0.2% BSA-PBS to produce a 10-ng μl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

Glycine buffer pH 2.4 Dissolve 15.01 g in 1,000 ml of distilled H_2O to produce a 0.2 M glycine solution. Dilute 10 ml of HCl (stock concentration 2 M) in 90 ml of distilled water to produce a 0.2 M HCl solution. Dilute 50 ml of 0.2 M glycine solution with 32.4 ml of 0.2 M HCl solution in 117.6 ml of distilled H_2O to produce a final 50 mM glycine buffer of pH 2.4. Use immediately.

Glycogen solution Dissolve 1,500 μg of glycogen in 1,500 μl of H_2O to produce a 1-mg ml^{-1} solution. Store at −20 °C for several months.

GSH solution Dissolve 0.384 mg of GSH in 1 ml of phosphate buffer to produce a 1.25 mM solution. Use immediately.

TABLE 3 | Expansion medium (500 ml).

Component	Volume (ml)	Final concentration
DMEM-low glucose	270	
MCDB	200	
FBS	10	2%
ITS	5	1×
LA-BSA	5	1×
Penicillin-streptomycin	5	1×
L-Ascorbic acid (10 mM)	5	100 nM
PDGF-BB (10 μg ml^{-1})	0.5	10 ng ml^{-1}
Dexamethasone (0.25 mM)	0.1	50 nM
LIF (10^7 units ml^{-1})	0.05	10^3 units ml^{-1}
EGF (200 μg ml^{-1})	0.025	10 ng ml^{-1}

EGF, epidermal growth factor; ITS, insulin-transferrin-selenium; LA-BSA, linoleic acid–BSA; LIF, leukemia inhibitory factor.

Hepatic differentiation medium (Table 4) Store at 4 °C for 1 month.
HGF solution Dissolve 25 µg (one vial) in 2,500 µl of 0.2% BSA-PBS to produce a 10-ng µl^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.
L-Ascorbic acid solution Dissolve 1.45 g in 500 ml of PBS to produce a 10 mM solution. Stir with a magnetic stir bar for 10–20 min in the dark and filter using Stericup filter. Store at −20 °C for several months.
Omeprazole solution Dissolve 40 mg (one vial) of omeprazole in 10 ml of 0.9% NaCl to produce a 4-mg ml^{-1} (11.58 mM) solution. Store at −20 °C for several months.
Platelet-derived growth factor BB solution (PDGF-BB) Dissolve 50 µg (one vial) in 5 ml of 0.2% BSA-HCl to produce a 10-µg ml^{-1} solution. Store at −80 °C for several months.
Phenobarbital solution Dissolve 5 g of phenobarbital in 21.52 ml of PBS to produce a 1 M stock solution. Store at −20 °C for several months. Dilute 500 µl of 1 M stock solution in 9.5 ml of PBS to produce a final 50 mM working solution. Store at 4 °C for several weeks.
Phosphate buffer pH 6.5 Dissolve 1.7 g of KH_2PO_4 in 100 ml of PBS to produce a 125 mM solution. Measure the pH and add drops of KOH until the pH is 6.5. Use immediately.
Wnt3a solution Dissolve 5 µg (one vial) in 500 µl of 0.2% BSA-PBS to produce a 10-ng ml^{-1} solution. Store at 4 °C for 1 week or at −80 °C for several months.

EQUIPMENT SETUP
Microplate reader (albumin secretion, Step 22A, and urea production, Step 22C only) Use dual optical photometric settings with 450 nm as the first wavelength (samples) and 655 nm as the second wavelength (background).
Spectrophotometer (glycogen storage, Step 22B only) Use the quantitative mode with a wavelength of 620 nm. Create a standard curve with 12 readings (six readings in duplicate), entering the micrograms of glycogen.
Spectrophotometer (GST activity, Step 22E only) Use the time-course mode with a wavelength of 340 nm. Set the number of readings to 7, with a 1-min interval.

TABLE 4 | Hepatic differentiation medium (500 ml).

Component	Volume (ml)	Final concentration
DMEM-low glucose	285	
MCDB	200	
Penicillin-streptomycin	5	1×
L-Ascorbic acid (10 mM)	5	100 nM
Dexamethasone (0.25 mM)	2	1 µM
ITS	1.25	0.25×
LA-BSA	1.25	0.25×
2-Mercaptoethanol (50 mM)	0.5	50 µM

ITS, insulin-transferrin-selenium; LA-BSA, linoleic acid–BSA.

Luminometer (cytochrome activity, Step 22D only) Start with a shaking step for 1 min at 300 r.p.m., followed by detection using the kinetic mode. Set the number of readings to 11, with a 2-min interval.
High-pressure liquid chromatography (bilirubin conjugation, Step 22F only) Create solvent A (66 ml of methanol + 39 ml of Milli-Q water + 160 µl of TBA, brought to pH 7.6 with H_3PO_4) and solvent B (75 ml of methanol + 16 ml of Milli-Q water + 10 ml of ethanol + 140 µl of TBA + 150 µl of Tris, brought to pH 7.5 with H_3PO_4) and connect both bottles to a HPLC pump. Set a continuous solvent flow rate of 1 ml min^{-1}. Start with 100% solvent A–0% solvent B mixture and gradually change to 0% solvent A–100% solvent B mixture by minute 11. Continue the same settings for 6 min until minute 17.

PROCEDURE

Seeding undifferentiated rMAPCs ● TIMING 1 h (1 day before differentiation)

1| Grow and isolate rMAPCs; for further details, see **Box 1** (ref. 1). Grow cells in 10-cm dishes until they are >80% confluent.

2| Aspirate the expansion medium and rinse the dishes with PBS.

3| To detach the cells, add 1 ml of trypsin 0.25%. Increase the dislodgment of cells by patting the plates gently. Check using a microscope whether all cells are detached.
▲ CRITICAL STEP Avoid overexposure to trypsin, as this is harmful to the cells. Limit the incubation time to less than 2 min, and do not incubate at 37 °C during trypsinization.

4| Block trypsin with 2 ml of expansion medium per dish.

5| Aspirate cells in a centrifuge tube and quantify using NucleoCounter.

6| Centrifuge at 700*g* for 6 min.

7| Remove the supernatant and resuspend 10^6 cells in 7.5 ml of expansion medium.

8| Remove 2% Matrigel solution from wells of a 12-well Matrigel-coated plate (prepared as detailed in REAGENT SETUP).

9| Add 1.5 ml of cell suspension to every well (200,000 cells per well, density 52,600 cells cm^{-2}).

10| Store in a 5% O_2–5.8% CO_2–37 °C incubator until cells become 80–90% confluent (usually after 12–24 h).
▲ CRITICAL STEP rMAPCs need to be confluent when initiating differentiation cultures, hence it is important not to start the first step of differentiation too quickly after plating the cells.

Hepatocyte differentiation protocol ● TIMING 20 days

11| Remove the expansion medium, rinse wells with PBS.

▲ **CRITICAL STEP** Ensure that floating cells are washed away carefully because they may have a negative impact on the first days of differentiation.

12| Add 1.5 ml of differentiation medium containing 15 µl of Activin-A solution (final concentration 100 ng ml^{-1}) and 7.5 µl of Wnt3a solution (final concentration 50 ng ml^{-1}) per well. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 2.

13| On day 2, remove 950 µl of medium and add 1,000 µl of differentiation medium containing 10 µl of Activin-A solution and 5 µl of Wnt3a solution. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 4.

14| On day 4, remove 950 µl of medium and add 1,000 µl of differentiation medium containing 10 µl of Activin-A solution and 5 µl of Wnt3a solution. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 6.

15| On day 6, remove all medium. Rinse with PBS. Add 1.5 ml of differentiation medium containing 1.5 µl of FGF2 solution (final concentration 10 ng ml^{-1}) and 7.5 µl of BMP4 solution (final concentration 50 ng ml^{-1}). Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 8. If desired, retain the samples for further analysis (see Step 22).

16| On day 8, remove 950 µl of medium and add 1,000 µl of differentiation medium containing 1 µl of FGF2 solution and 5 µl of BMP4 solution. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 10.

17| On day 10, remove all medium. Rinse with PBS. Add 1.5 ml of differentiation medium containing 3 µl of FGF1 solution (final concentration 20 ng ml^{-1}), 1.5 µl of FGF8b solution (final concentration 25 ng ml^{-1}) and 0.6 µl of FGF4 solution (final concentration 10 ng ml^{-1}). Store in 21% O_2–5.8% CO_2–37 °C incubator until day 12. If desired, retain samples for further analysis (see Step 22).

18| On day 12, remove 950 µl of medium and add 1,000 µl of differentiation medium containing 2 µl of FGF1 solution, 1 µl of FGF8b solution and 0.4 µl of FGF4 solution. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 14.

19| On day 14, remove all medium. Rinse with PBS. Add 1.5 ml of differentiation medium containing 3 µl of HGF solution (final concentration 20 ng ml^{-1}) and 15 µl of Follistatin-288 solution (final concentration 100 ng ml^{-1}). Store in 21% O_2–5.8% CO_2–37 °C incubator until day 16. If desired, retain the samples for further analysis (see Step 22).

20| On day 16, remove 950 µl of medium and add 1,000 µl of differentiation medium containing 2 µl of HGF solution and 10 µl of Follistatin-288 solution. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 18.

21| On day 18, remove 950 µl of medium and add 1,000 µl of differentiation medium containing 2 µl of HGF solution and 10 µl of Follistatin-288 solution. Store in a 21% O_2–5.8% CO_2–37 °C incubator until day 20.

22| On day 20, analyze the hepatocyte characteristics. A single test cannot effectively evaluate the hepatocyte characteristics of MAPC progeny. It is important to assess the gene expression profile, as well as protein synthesis and functional analysis, of the cells. This can be carried out in a number of ways: real-time RT-qPCR (**Box 2**), immunocytochemistry (**Box 3**), ultrastructural evaluation of hepatocyte cells (electron microscopy, data not shown) or by functional analysis (options A–F) of albumin secretion (option A), glycogen storage as per the spectrophotometrical method of Seifter *et al.*[24] (option B), urea production (option C), determination of cytochrome P450 subtype Cyp1a1/1a2 activity (option D), determination of GST activity according to the spectrophotometrical method of Habig *et al.*[25] (option E) and/or bilirubin conjugation by HPLC according to the method of Muraca and Blanckaert[26] (option F).

(A) Albumin secretion ● **TIMING 5 h**

(i) Collect the supernatant from desired time points in 1.5-ml tubes.

(ii) Centrifuge at >8,000*g* for 1 min.

■ **PAUSE POINT** Use immediately or store at −20 °C.

(iii) Proceed as indicated by the manufacturer (step 1, capture the antibody for 60 min; step 2, use the blocking solution for 30 min; step 3, dilute the samples at 1:40 and use the standard solution for 60 min; step 4, use the HRP detection antibody (part of the Rat Albumin ELISA Quantitation Set (see REAGENTS)) for 60 min; step 5, TMB (TMB-peroxidase solution; part of the ELISA Starter Accessory Kit (see REAGENTS)) for 15 min; step 6, H_2SO_4).

(iv) Read the OD on a microplate reader at a wavelength of 450 nm (specific for TMB). Determine the average OD of duplicate wells and subtract the OD_{medium} from all samples.

(v) Create a four-parameter standard curve ($y = ax^3 + bx^2 + cx + d$) by plotting $OD_{standard}$ on the x axis and $\log(Conc_{standard})$ on the y axis (log scale is used to ensure that no negative values are obtained).

BOX 3 | IMMUNOCYTOCHEMISTRY EVALUATION OF PROTEIN EXPRESSION

(i) Fix the cells in wells using 10% neutral buffered formalin (NBF)
(ii) Permeabilize cells with PBST (0.2% Triton in PBS) containing 3–5% serum of the secondary antibody for blocking. Alternatively, donkey serum can be used.
(iii) Incubate overnight at 4 °C with primary antibodies and the respective isotypes (diluted in PBST containing 3–5% serum).
(iv) Incubate with secondary antibodies and nuclear counter stain
(v) Mount slides and store at 4 °C in the dark
(vi) Possible combinations are (items in boldface type are detailed in **Fig. 4**)
Undifferentiated rMAPCs: **Oct4$^+$/Foxa2$^+$/Sox7$^+$/Sox17$^+$**
Hepatoblast and fetal hepatocyte stage: Oct4$^-$/Afp$^+$/Alb$^+$, **Krt18$^+$**
More mature hepatocyte-like cells: **Afp$^-$/Alb$^+$**, Aat$^+$, Mrp2$^+$, **Pepck$^+$**
Liver-enriched transcription factors: Hnf4α^+

Adapted from Sancho-Bru *et al.*[28].

(vi) Calculate log($Conc_{sample}$) by using OD_{sample} in the standard curve equation.
(vii) To obtain the final concentration, calculate 10 to the power log($Conc_{sample}$) and multiply by 40 (dilution correction). Results are depicted as ng ml^{-1}.

Standard curve: $y = ax^3 + bx^2 + cx + d$

$$Albumin_{sample} = 40*10\,\text{^}[a*(OD_{sample})^3 + b*(OD_{sample})^2 + c*(OD_{sample}) + d]$$

(B) Glycogen storage ● TIMING 3–4 h

(i) At the desired time point, remove the supernatant from the wells of interest and rinse with PBS. If required, different concentrations of glucose in the medium can be added, as this will influence the amount of stored glycogen (see Anticipated results).
(ii) Add 500 µl of trypsin 0.05% until the cells detach.
(iii) Add 1,000 µl of medium/supernatant to block trypsin.
(iv) Scrape the bottom of wells with the tip of a 1,000-µl pipette or cell scraper to ensure that all cells are collected.
(v) Pipette the cell suspension in a 1.5-ml tube and centrifuge at 2,100*g* for 6 min.
(vi) Remove the supernatant and resuspend the pellet in 200 µl of distilled H_2O to create a homogenous cell suspension.
■ **PAUSE POINT** Samples can be stored at –80 °C for several weeks.
(vii) Pipette 60 µl of the cell suspension in a 1.5-ml tube with a screw top. Store the remaining cell suspension at –20 °C for protein analysis.
(viii) Add 240 µl of 33% KOH solution to the tube and screw the top tightly.
(ix) Heat the tube to 100 °C in heating block for 20 min.
(x) Cool the tube in ice water.
(xi) Pipette 125 µl of the sample-KOH suspension in a 15-ml tube in duplicate.
(xii) Pipette 875 µl of H_2O to each 15-ml tube (total volume 1 ml).
(xiii) Prepare the standard curve by bringing 0, 1, 2, 3, 5 and 10 µl of a 1-mg ml^{-1} glycogen solution to a total volume of 1 ml with H_2O in a 15-ml tube.
(xiv) Slowly pipette 2 ml of yellow anthrone solution to all tubes.
! CAUTION Adding H_2SO_4 to H_2O results in a strong exothermic reaction, which may spatter if added too quickly. The bottom of the tube will heat up and may cause burns. Wear protective glasses, clothes and gloves.
(xv) Vortex the tubes. The color of the standard curve changes from yellow to dark green. Because of the reaction with glycogen, the solution is slightly viscous (the darker the color, the higher the viscosity, and the higher the glycogen content).
(xvi) Leave for 10 min, vortex again.
(xvii) Read the OD at 620 nm (1 ml per cuvette) starting with a standard curve with known content (0–1–2–3–5–10 µg). The readout of the samples provides you with the glycogen content in the tube (µg).
(xviii) Multiply the glycogen content by 40 (correction for KOH and H_2O–anthrone dilution) to obtain the glycogen concentration in the primary cell suspension (µg ml^{-1})

Standard curve: $y = ax + b$

$$Glycogen_{sample} = 40*[a*OD_{sample} + b]$$

(xix) To compare samples, quantify the total protein content by using the spectrophotometrical method of Bradford *et al.*[27]. Alternative methods can be used.

(xx) Divide the glycogen concentration (μg ml^{-1}) by the protein concentration (mg ml^{-1}). The results can be displayed as μg glycogen per mg protein or as nmol glucose per mg protein (dividing the glycogen concentration by the molecular mass of glucose, 180.16 g mol^{-1}).

(C) Urea production in relation to ammonia ● TIMING 1 h plus 24 h incubation before assay

(i) At the desired time point, remove the supernatant from the wells of interest and wash the wells with PBS.

(ii) Add 1,000 μl of differentiation medium containing 0 or 1 mM of ammonium bicarbonate (NH_4HCO_3). Use one well containing 1 mM of sodium bicarbonate ($NaHCO_3$) as control for increased alkalosis.
▲ **CRITICAL STEP** Do not use NH_4Cl, as this will make the medium slightly more acidic. Acidosis inhibits the production of urea. If NH_4HCO_3 is used, always use 1 mM of $NaHCO_3$ in the control well to normalize for the relative alkalosis.

(iii) After 24 h, collect the supernatant in a 1.5-ml tube and replace with normal differentiation medium.
■ **PAUSE POINT** Use immediately or store samples at −20 °C until further use.

(iv) Transfer 50 μl of supernatant in duplicate to a transparent 96-well plate.

(v) Dilute the internal standard to 1:10 with H_2O to a final concentration of 5 μg ml^{-1}. Transfer 50 μl of internal standard in duplicate to a transparent 96-well plate.

(vi) Transfer 50 μl of fresh hepatic differentiation medium in duplicate to a transparent 96-well plate.

(vii) Mix an equal volume of QuantiChrom Urea Assay Kit Reagents A and B.

(viii) Add 200 μl of reagent mix to every well.

(ix) Transfer 50 μl of supernatant in duplicate to a transparent 96-well plate.

(x) Incubate at room temperature for 50 min.
▲ **CRITICAL STEP** Unlike with classical ELISA, there is no termination of the reaction. Ensure that absorbance is read no later than 50 min after adding the reagents.

(xi) Read the absorbance at 450 nm.

(xii) Subtract the result of the medium from every sample. Divide by absorbance of the internal standard and multiply by 5 to obtain the final result, which is given in μg ml^{-1}.

$$Urea_{sample} = [(Abs_{sample} - Abs_{medium}) / Abs_{internal\,standard}] * 5$$

(D) Cytochrome P450 activity ● TIMING 6 h plus 24 h incubation before assay

(i) To study the induction at a desired time point, add 10 μM of omeprazole (1.3 μl of stock solution) or 500 μM of phenobarbital (10 μl of working solution) to the differentiation medium 24 h before the assay to the wells to be evaluated. As omeprazole is stable only for 12 h, repeat the process 12 h before the assay. Always use one well as negative control by adding 1.3 or 10 μl of PBS, respectively, to the differentiation medium.

(ii) At the desired time point, remove the supernatant and rinse well with PBS.
▲ **CRITICAL STEP** Wash well because omeprazole and phenobarbital degradation products may interfere with the luciferase assay.

(iii) Pipette 400 μl of hepatic differentiation medium into every well, including one well without cells (background luminescence).

(iv) Add 8 μl of luminogenic substrate (Luciferin-ME specific for Cyp1a1/1a2) to every well.

(v) Store in 21% O_2–5.8% CO_2–37 °C incubator for 4 h.

(vi) Collect supernatant, replace with 500 μl of medium (to keep the cells from drying).

(vii) Transfer 50 μl of supernatant to a white opaque 96-well plate in triplicate.

(viii) Add 50 μl of Luciferin Detection Reagent to every well.

(ix) Read the luminescence (11 consecutive time points with a 2-min interval, total 22 min). In the meantime, remove all medium and add 500 μl of trypsin 0.05% to the wells until the cells detach.

(x) Add 1,000 μl of the medium or supernatant to block the trypsin.

(xi) Scrape the bottom of wells with the tip of a 1,000-μl pipette or cell scraper to ensure that all cells are collected.

(xii) Count the cells.

(xiii) Determine the average signal of 11 time points ($Lumin_{sample}$) and subtract $Lumin_{medium}$ from the samples to obtain the net signal.

(xiv) To normalize for the different cell number, multiply the net signal by 10^6 and divide by the number of cells. Results are now depicted as cytochrome activity per million cells. As the results are presented in relative light units, scaling can be performed easily. In induction studies, results can also be depicted as fold increase.

$$Cyp1a2\ activity_{sample} = 1{,}000{,}000 * (Lumin_{sample} - Lumin_{medium}) / cell\,number$$

$$Increase = activity_{day\,20} / activity_{day\,14} \text{ or } activity_{induction} / activity_{no\,induction}$$

(E) GST activity ● TIMING 1 h

(i) At the desired time point, remove the supernatant from the wells of interest and rinse with PBS.

(ii) Add 500 µl of trypsin 0.05% until the cells detach.
(iii) Add 1,000 µl of medium/supernatant to block trypsin.
(iv) Scrape the bottom of the wells with the tip of a 1,000-µl pipette to ensure that all cells are collected.
(v) Collect the cell suspension in a 1.5-ml tube and centrifuge at 2,100*g* for 6 min.
(vi) Remove the supernatant and resuspend the pellet in 300 µl of PBS to create a homogenous cell suspension.
■ **PAUSE POINT** Samples can be stored at −80 °C for several weeks.
(vii) Add 600 µl of GSH solution and 25 µl of CDNB solution to the cuvette.
(viii) Add 125 µl of cell suspension to the cuvette, mix well. Use 125 µl of PBS as negative control.
(ix) Read the absorbance at 340 nm (formation of the CDNB-GSH conjugate) for 6 min with a 1-min interval (seven readings).
(x) Only the linear part of the reaction should be used to calculate the GST activity. Therefore, subtract the absorbance at minute 2 (Abs_{2min}) from the absorbance at minute 6 (Abs_{6min}) to define the difference in absorbance (ΔAbs).
(xi) Subtract ΔAbs_{PBS} from ΔAbs_{sample}.
(xii) Divide ΔAbs by 4 (ΔAbs per minute) and divide by the extinction coefficient of CDNB, namely, 9.6 mM^{-1} (results depicted as µM per min GSH-CDNB). Multiply by 750 to obtain the activity as nmol per min GSH-CDNB

$$\Delta Abs_{sample/PBS} = Abs_{sample/PBS6min} - Abs_{sample/PBS2min}$$

$$\text{Total GST activity}_{sample} = 750\,ml * [(\Delta Abs_{sample} - \Delta Abs_{PBS})/4]/9.6\,mM^{-1}$$

(xiii) To compare samples, quantify the total protein content using either the spectrophotometrical method of Bradford[27] or alternative methods.
(xiv) Divide the amount of GSH-CDNB by the protein concentration (mg ml^{-1}). The results can be displayed as nmol GSH-CDNB per mg protein per min.

(F) Bilirubin conjugation ● TIMING 2–3 h (up to 33 h of sample harvesting)

(i) At the desired time point, remove the supernatant from the wells of interest and rinse with PBS.
(ii) Add 1,000 µl of bilirubin solution to the wells of interest and to one empty well (first negative control). Add 1,000 µl of 60-µM BSA solution to one well containing cells as a second negative control.
▲ **CRITICAL STEP** As bilirubin is light sensitive, it is important to work in a dim-light environment.
(iii) Harvest the supernatant at different time points (6, 12, 24 and 33 h).
■ **PAUSE POINT** Samples can be stored at −80 °C for a maximum of 1 month.
(iv) Place 500 µl of sample in 10-ml glass tubes with ground-glass stoppers.
(v) Add 60 mg of ascorbic acid and 2 mg of disodium EDTA.
(vi) Add 2 ml of internal standard solution (xanthobilirubinic acid methyl ester 2 µg ml^{-1}).
(vii) Add 6 ml of 2% KOH solution.
(viii) Vortex and incubate the mixture for 60–90 s.
(ix) Add 6 ml of chloroform and 12 ml of glycine buffer.
(x) Shake briefly and centrifuge at 1,000*g* for 10 min.
(xi) Transfer the organic phase (bottom chloroform layer) to a dry 10-ml conical tube.
(xii) Evaporate at 50 °C under a N_2 stream.
■ **PAUSE POINT** The residue can be stored at −20 °C for 2 weeks.
(xiii) Dissolve the residue in 10 µl of chloroform and 100 µl of DMSO shortly before analysis, shake briefly.
(xiv) Inject 10 µl into the liquid chromatograph. Separate the pigments by eluting the column at a solvent rate of 1 ml min^{-1} with a linear gradient over 17 min. Monitor the absorbance at 436 nm.
(xv) In case of doubt (for example, heme interference in control samples), analyze the different peaks by using spectrum analysis. The internal standard has a peak emission at 413 nm, bilirubin around 450 nm and heme at 405 nm.
(xvi) Normalize the equation for different volumes (volume internal standard 2 ml, volume of the sample 0.5 ml) and compare with the concentration of the internal standard (2 µg ml^{-1}). To carry this out, multiply the equation of the areas by 8 and by the pigment-dependent factor F. Depict results as µg ml^{-1}. The results can also be depicted as % decrease in unconjugated bilirubin or % conjugation of bilirubin into mono/diconjugate.

$$\text{Bilirubin pigment concentration} = 8 * F * A_{pigment} / A_{IS}$$

where $A_{pigment}$ is the peak area of the pigment, A_{IS} the peak area of internal standard, F the pigment-dependent factor, unconjugated bilirubin $F = 1.102$, bilirubin monoconjugate $F = 1.279$, bilirubin diconjugate $F = 1.329$.

● **TIMING**

Steps 1–10, Seeding undifferentiated rMAPCs: 1 h (1 day before differentiation)
Steps 11–21, Hepatocyte differentiation: 20 days

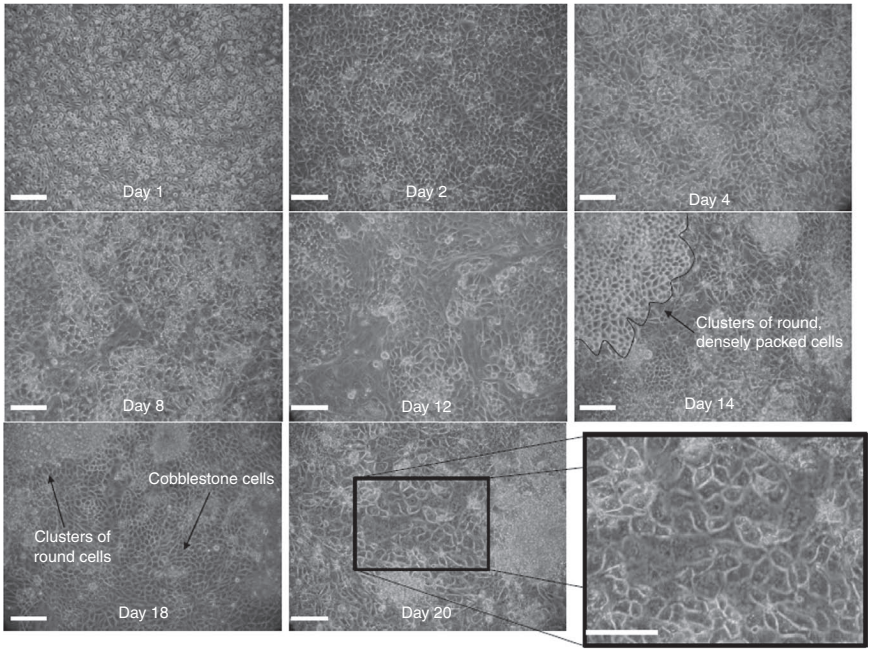


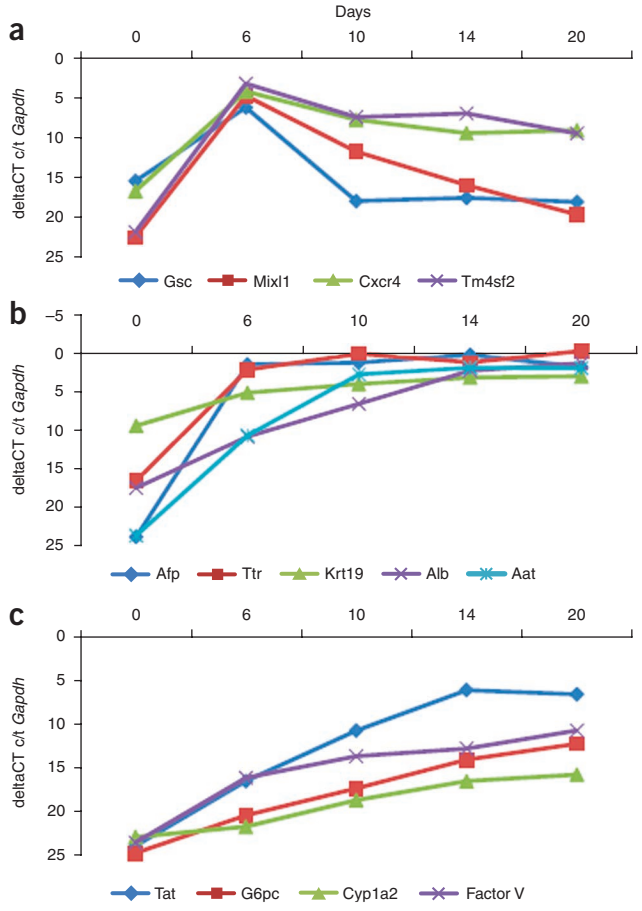
Figure 2 | Bright field microscopy of rMAPC progeny during liver differentiation. Undifferentiated rMAPCs are round to spindle shaped. On plating at high density they become more triangular and square (days 2–4). During the later stages of differentiation, clusters of round, densely packed cells appear together with more cobblestone-like cells. By day 20, the cells are more rectangular with clear nuclei (scale bar, 100 μm).

Step 22 option A, Albumin secretion: 5 h
Step 22 option B, Glycogen storage: 3–4 h
Step 22 option C, Urea production: 1 h (24-h incubation before assay)
Step 22 option D, Cytochrome P450 activity: 6 h (24-h incubation before assay)
Step 22 option E, GST activity: 1 h
Step 22 option F, Bilirubin conjugation: 2–3 h (up to 33 h of sample harvesting)

ANTICIPATED RESULTS

To ensure that differentiation has been performed correctly, it is important to monitor the acquisition of the correct phenotype for each of the embryonic stages of liver differentiation. Morphological changes can be visualized easily with bright field microscopy (**Fig. 2**).

As indicated in the detailed protocol section, samples are taken for RNA analysis and functional assays at 6, 10, 14 and 20 days. No single gene or test can efficiently identify the phenotype acquired by the stem cell progeny. In **Boxes 2** and **3**, an extended list of assays that allow identification of hepatocyte-like cell differentiation is summarized.



Gene expression profile of stem cell progeny

Gene expression analysis can be performed on days 0, 6, 10, 14 and 20 of differentiation to identify the correctly directed differentiation of the cells. Step 1 of the differentiation protocol clearly induces the expression of PS/ME/DE genes such us *Gsc*, *Mixl1*, *Cxcr4* and *Tm4sf2* (**Fig. 3a**). After stimulating first with BMP4 and FGF2 (Step 2), and then with FGF1, 4 and 8 (Step 3), those genes that are typically expressed in hepatoblasts and immature hepatocytes begin to be expressed (*Afp*, *Ttr*, *Krt19*, *Alb*, *Aat*) (**Fig. 3b**). Step 4 induces the maturation of immature liver cells to a hepatocyte-like phenotype, which is revealed by the increased expression of genes expressed perinatally such as *Tat*, *G6pc*, *Cyp450* and *Factor V* (**Fig. 3c**). Besides commitment to the liver, a fraction of cells is also directed to other endodermal (*Pdx1*, *Cdx2*, *Tmprss2*) and mesodermal cells (*Sm22*, *α-Sma*, *Cnn1*, *Nkx2.5*, *Tbx5*).

Immunocytochemistry analysis shows that at day 0 nearly 100% of the cells are $Oct4^+$ $Sox7^+$ $Sox17^+$ $Foxa2^+$

Figure 3 | RT-qPCR analysis of rMAPC progeny during liver differentiation, depicted as deltaCT compared with *Gapdh* (DeltaCT = $CT_{gene} - CT_{GAPDH}$). The lower the DeltaCT value, the higher the expression level. (**a**) Induction of primitive streak/mesendoderm/definitive endoderm genes. (**b**) Induction of early liver-specific genes. (**c**) Induction of more mature liver-specific genes.

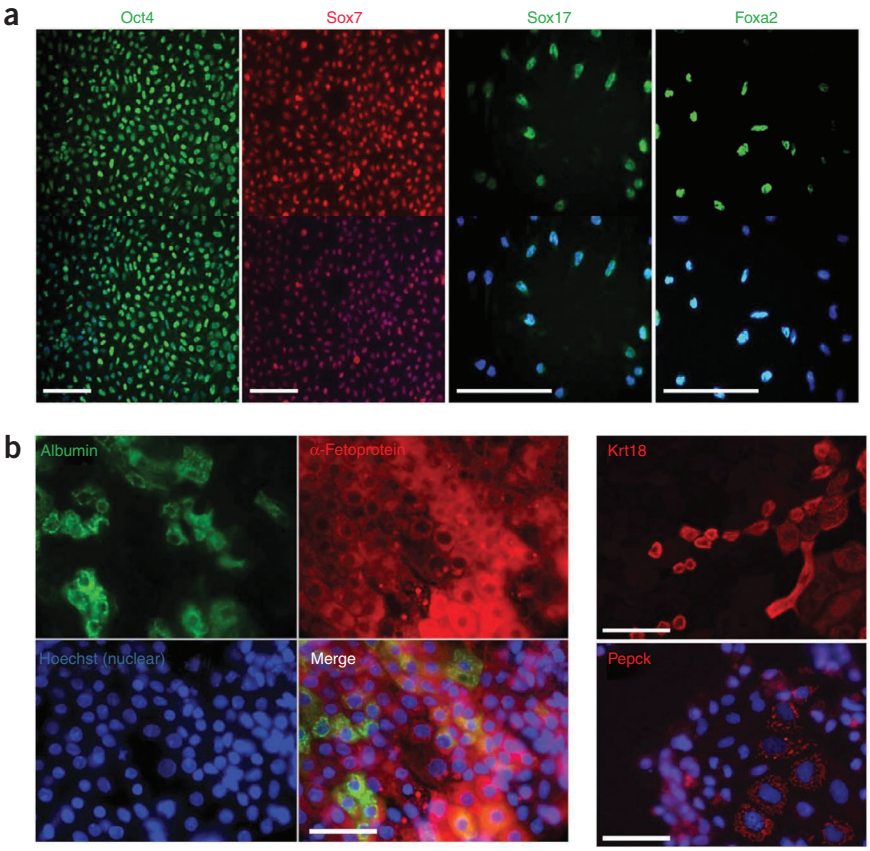


Figure 4 | Immunocytochemistry analysis. (**a**) Undifferentiated rMAPCs are positive for Oct4, Sox7, Sox17 and Foxa2 (scale bar, 100 μm). (**b**) Day 20 rMAPC progeny express albumin (green), α-fetoprotein (red) and to a lesser extent Krt18 and Pepck (scale bar, 50 μm).

(**Fig. 4a**). At day 20, the differentiated stem cell progeny is a mixture of immature cells that coexpress Alb and Afp, and more mature cells expressing Alb but no longer Afp. Approximately 80% of differentiated cells expressed Afp, whereas expression of Alb was variable and patchy (range 5–30% Alb^+). A small fraction of the progeny is $Krt18^+$ and $Pepck^+$ (**Fig. 4b**). Electron microscopy studies of the differentiated population reveal cells with immature characteristics, together with cells with more mature hepatocyte characteristics (data not shown).

Hepatocytes exert a number of synthetic, storage and detoxifying functions. Thus, functional assays allow a more stringent characterization of stem cell progeny. As shown in **Figure 5a,b**, differentiated cells produce albumin and store glycogen (in response to the glucose level in the medium). Moreover, stem cell progeny secrete urea, which is enhanced by the incubation with NH_4HCO_3 (**Fig. 5c**), and have inducible cytochrome activity, as incubation of cells with omeprazole (as well as with phenobarbital, a well-known inducer of Cyp1a2) results in increased activity (**Fig. 5d**). Finally, hepatocyte-like cells are able to detoxify as demonstrated by GST activity (**Fig. 5e**) and bilirubin conjugation (**Fig. 5f**).

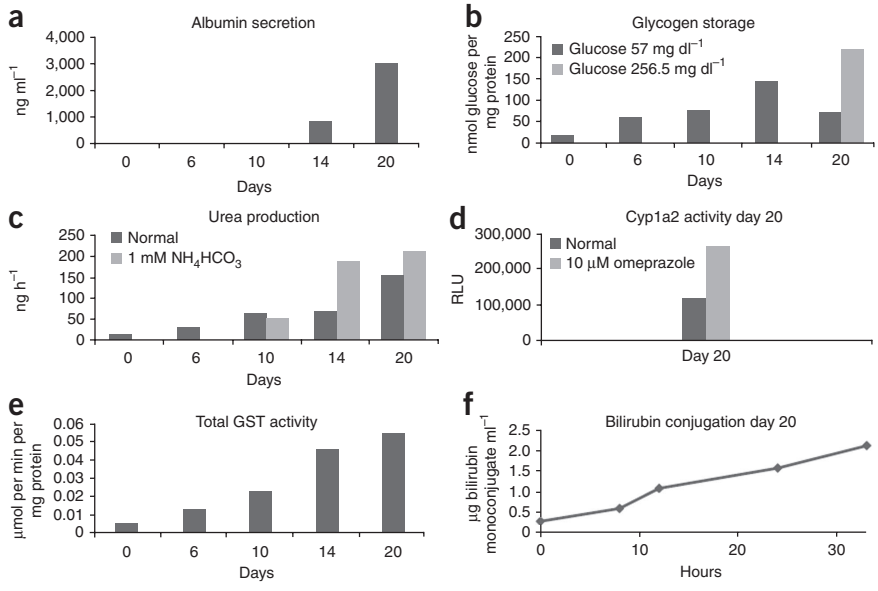


Figure 5 | Functional assays on rMAPC progeny (one representative experiment) during liver differentiation. (**a**) Albumin secretion; (**b**) glycogen storage in relation to low and high concentrations of glucose; (**c**) urea production in relation to ammonia; (**d**) rMAPC d 20 progeny Cyp1a2 activity, inducible with omeprazole; (**e**) GST activity; and (**f**) rMAPC d 20 progeny bilirubin conjugation.

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